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Cellular signals of cisplatin ototoxicity

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CELLULAR SIGNALS OF CISPLATIN OTOTOXICITY

by

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Abstract: ***Three separate theories of inhibiting cisplatin-induced apoptosis were investigated utilizing different cellular mechanisms. Specifically, the copper transport cycle, TRPV1, and the JNK pathway were inhibited and immunohistochemistry was performed to determine levels of apoptosis. All three resulted in statistically significant effects; increasing $CuSO_4$ levels resulted in increased apoptosis, and inhibiting TRPV1 and JNK resulted in decreased apoptosis.***

Acknowledgements

The following individuals deserve recognition for supporting me throughout this project:

Dr. Mark Warchol: For sharing your time and lab with me, answering countless questions, teaching me numerous skills and concepts, and overall helping me complete this capstone.

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My friends and family: For supporting me throughout the years, but especially throughout the past year.

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Background

Cis-diamminedichloroplatinum, or cisplatin, is an antineoplastic agent commonly used for the treatment of a variety of cancers; including testicular, ovarian, bladder, cervical, head and neck, and non-small cell lung cancers. The dose-limiting effects of cisplatin are well-documented and include nephrotoxicity, emetogenesis, and ototoxicity (Wang & Lippard, 2005; Rybak et al., 2007). Ototoxic effects (such as tinnitus and high-frequency hearing loss) have been reported in approximately 30% of treated individuals (Nagy et al., 1999). It has been demonstrated that cisplatin not only promotes apoptosis in auditory cells (van Ruijven et al., 2005), but also vestibular cells (Slattery & Warchol, 2010). While much research has been conducted on the mechanisms of cisplatin ototoxicity, it remains a significant clinical problem. There continues to be a need for refinement and additional research on the cellular uptake and method of action of cisplatin

Copper Uptake as a factor in ototoxicity

Studies have shown that cisplatin-resistant cell lines exhibit a cross-resistance to copper efflux, indicating a potential connection between copper transporters and cisplatin intake in cells (Matsumoto et al., 2007; Kuo et al., 2007; Howell, 2010). Copper is an important micronutrient, and copper transporters facilitate copper introduction into cells. Copper transporters 1 & 2 (CTR1, CTR2) are generally thought to be copper's main method of entry into the cell (Blair et al., 2009). Notably, CTR1 and CTR2 are also thought to transport cisplatin into cells (Kuo et al., 2007; Blair et al., 2009; Howell, 2010). ATP7A and ATP7B are copper transporters that transport copper out of cells. Rabik et al. (2009) demonstrated increased activation of ATP7A and ATP7B appeared to correlate with increased removal of cisplatin out of cells. Both Matsumoto et al. (2007) and Holzer et al. (2004) found that treatment with $CuSO_4$ (copper

sulfate) leads to increased cellular uptake of cisplatin, particularly in cisplatin-resistant cell lines. Published studies have investigated the interactions of $CuSO_4$ and cisplatin primarily in laboratory-generated human cell lines. Matsumoto et al. (2007) utilized human epidermoid cancer cell lines with varying amounts of cisplatin-resistance, while Holzer et al. (2004) investigated the affect of $CuSO_4$ administration on ovarian carcinoma cell lines. It is proposed that investigating the role of $CuSO_4$ on cisplatin ototoxicity *in vitro* using the utricular epithelium of post-hatch chicks will provide a more controlled testing environment.

Inhibition of vanilloid receptors

Capsaicin is known to activate vanilloid receptors, which have been identified in the inner ear (Zheng et al., 2003). Vanilloid receptor Transient Receptor Potential Vanilloid 1 (TRPV1) is expressed in hair and supporting cells in the organ of Corti. It has been shown that TRPV1 is activated by Reactive Oxygen Species (ROS) formation, which has been linked to ototoxicity (Clerici et al., 1995; Mukherjea et al., 2008). Vanilloid receptors can be blocked using capsazepine, an antagonist to capsaicin. Mukherjea et al. (2008) found that the inhibition of TRPV1 using the antagonist capsazepine reduced the ototoxic effects of cisplatin administration, thus suggesting a link between activation of TRPV1 and cisplatin ototoxicity. However, that study used an immortalized cell line *in vitro*, as well as living rats. It is believed that performing an *in vitro* study using utricle epithelium from post-hatch chicks will provide a more realistic and controlled basis for evaluating the effect of TRPV1 inhibition on the ototoxic effects of cisplatin.

Regulation of apoptosis through MAPKs

Zine & van de Water (2004) note the importance of identifying regulatory components of apoptosis to better develop methods of preventing cell death. Mitogen activated protein kinases (MAPKs) have been identified as regulators of apoptosis (Gallo & Johnson, 2002; Zine & van de Water, 2004; Yang et al., 1997) and are comprised of c-Jun amino-terminal kinases (JNK), p-38 kinases, and extracellular-signal-regulated kinases (ERK) (Bozyczko-Coyne et al., 2002). Many studies have shown that JNK activation leads to increased apoptosis in damaged cells (Eshraghi et al., 2007; Zine & Van De Water, 2004; Marderstein et al., 2003; Ylikoski et al., 2001; Pirvola, 2000; Tournier et al., 2000; Yang et al., 1997). JNK has been reported to phosphorylate and activate the transcription factor c-Jun (Derijard et al., 1994). Behrens et al. (1999) have identified c-Jun as being the mitigating factor in apoptosis in JNK-activated cells for kainite-induced apoptosis and Scarpidis et al. (2003) determined the JNK/c-Jun pathway has a direct relationship with apoptosis. In the present study, we hypothesize that c-Jun plays a vital role in the initiation of cellular apoptosis following cisplatin-ototoxicity.

Purpose of present study: The present study tests three previously proposed facets of cisplatin ototoxicity at the cellular level. Specifically, we examined the copper transport cycle with administration of $CuSO_4$, the role of the TRPV1 gene via inhibition with capsazepine, and the interaction with c-Jun by the inhibition of the JNK pathway.

Methods and materials

Preparation of cultures: Epithelial cultures were prepared by Judy Speck in Dr. Mark Warchol's laboratory. All protocols were approved by Washington University Institutional Animal Research Committee (Saint Louis, MO). In brief, post-hatch (D7-21) chickens (Charles River

Laboratories) were euthanized utilizing CO_2 asphyxiation and decapitated. Surface pathogens were removed using 70% EtOH for 5-10 minutes, and utricles were removed from exposed temporal bones and placed in chilled Medium 199 (M199) with Hanks' salts and HEPES buffer. Utricles were then transferred to Thermolysin (500 μg/ml in M199) and incubated for 1 hour at 37°C, then replaced with M199. Otoconia were removed at this time. Cultures were then transferred to culture wells (MatTek) and the epithelial layer was removed using a syringe needle. Earles M199 was placed in each well to cover culture, and cultures were incubated and fed fresh M199 for a period of 2-3 days.

Immunohistochemistry: Prepared cultures were treated according to the specific needs of the study. For experiments involving the copper pathway, a solution of M199 with 10% Fetal Bovine Solution (FBS) with 20 μM cisplatin was added to all cultures. $CuSO_4$ (1:50 at 2.5mM) was also immediately added to test cultures, though omitted from control cultures. Experiments involving TRPV1 required the treatment of a solution containing capsazepine. Medium 199 with 10% FBS with capsazepine (1:10) and Dimethyl sulfoxide (DSMO) (1:10) was further diluted, resulting in a final concentration of 10 μM capsazepine or 0.1% DSMO with medium 199. Cultures were treated with either this mixture or one containing no capsazepine (controls) and allowed to incubate for 30 minutes. After this period, 2 μL of concentrated cisplatin was added to all treated cells. Experiments that examined inhibition of the c-Jun pathway used a solution consisting of medium 199 with 10% FBS with 15μM SP600125 (dissolved in DSMO). Cultures were treated with either this mixture or one containing no SP600125 and allowed to incubate for 30 minutes before concentrated cisplatin (1:50) was added to all cells.

To determine the effects of the various inhibitors on cisplatin toxicity, we calculated an apoptotic index (AI) for the various cultures. The AI is defined as the proportion of cells

undergoing programmed cellular death following injury. Cell death was assessed using immunohistochemistry. Following the various treatments, the cultures were incubated for an additional 24 hours and then fixed with 4% paraformaldehyde (20 minutes at room temperature) and rinsed with phosphate buffered saline (PBS) 5 times over 15 minutes. Cultures were treated in a blocking solution of PBS with 0.2% Triton X-100 and 5% normal horse serum (NHS) for 2 hours prior to exposure to primary antibodies. Hair cells were identified with the HCS-1 antibody and apoptotic cells were labeled via immunoreactivity for activated caspase-3. A solution consisting of PBS combined with 0.2% Triton, Mouse IgG HCS-1 Otoferlin (1:500), Rabbit IgG Activated Caspase-3 (1:100), and 2% NHS were added to each well of cultures. After being incubated for 24 hours at 4°C, the solution was removed and a solution consisting of PBS combined with 0.2% triton X-100 and secondary antibodies Alexa 488 (1:500) (for hair cell identification), Cy3 (Anti-rabbit) (1:500) (for apoptotic cell identification), and DAPI (1:500) (for identification of cell nuclei) was added to each well for a period of 2 hours at room temperature. After rinsing 5 times over 15 minutes with PBS, mounting solution (glycerol with PBS (1:10)) was added and each well was cover-slipped.

Prior to investigating the effects of SP600125 on cisplatin-treated chick utricular cultures, we characterized the activation of the three MAPK pathways (JNK, p38, and ERK) in response to cisplatin treatment. This was accomplished by treating prepared cultures with medium 199 with 10% FBS and 20 μM of concentrated cisplatin. Control cultures were treated with only medium 199 with 10% FBS. After 24 hours of incubation, cultures were then fixed with 4% paraformaldehyde (20 minutes at room temperature) and rinsed with PBS 5 times over 15 minutes. Cultures were treated in a blocking solution of PBS with Triton X-100 (1:500) and NHS for 2 hours prior to exposure to primary antibodies. Following blocking, a solution consisting of

PBS combined with Triton (1:500), Rabbit IgG Activated Caspase-3 (1:100), NHS (1:50), and either Rabbit mAb p44/42 (ERK1/2) (1:500), Rabbit Ab P-p38 (1:500), or Rabbit Ab P-c-Jun (S63) (1:500) were added to each well of cultures, depending on intended investigation. Following incubation for 24 hours at 4°C, each solution was removed and a solution consisting of PBS combined with triton X-100 (1:500) and secondary antibodies Alexa 488 (1:500), Cy3 (Anti-rabbit) (1:500), and DAPI (1:500) was added to each well for a period of 2 hours at room temperature. After rinsing 5 times over 15 minutes with PBS, mounting solution (glycerol with PBS (1:10)) was added and each well was cover-slipped.

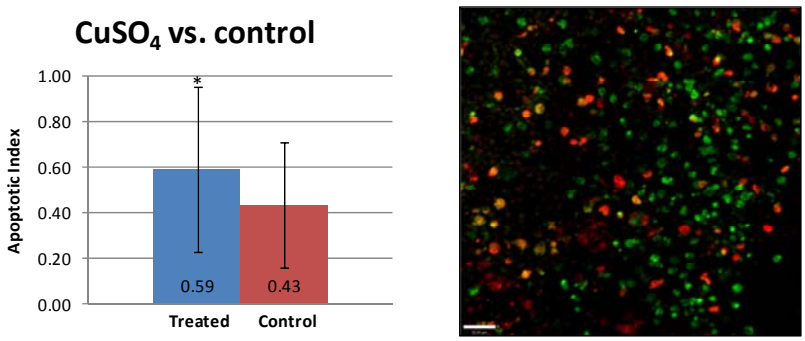
Imaging: Following immunohistochemical labeling, imaging was completed using a Zeiss Confocal microscope (LSM700) with a 20x objective. Two separate images identified through DAPI labeling were obtained utilizing Z-stacking per well of prepared cultures.

Quantification: Processed specimens were imaged using the Volocity imaging program. Each image's contrast was manually enhanced prior to quantification. Hair cell AI was determined by manual quantification of Cy3-labeled cells that were also labeled for Alexa 488. Total AI was determined by quantification of Cy-3 labeled cells as well as cell nuclei labeled with DAPI. Data were organized in a spreadsheet using Microsoft Excel and statistical analyses were performed.

Statistics: Significance was identified by utilizing a Student *t*-Test. Standard deviations were noted and results were displayed graphically. Computations were performed using Microsoft Excel.

Results

Administration of $CuSO_4$ to cisplatin-treated cultures of chick utricle epithelium significantly increased the apoptotic index (AI) of hair cells. The mean AI for $CuSO_4$ treated cells was 0.59 ±0.28, while the mean AI for control cultures was 0.43 ±0.36 (Figure 1, images 1 & 2). A Student's t-test demonstrated statistical significance at the <0.05 level.



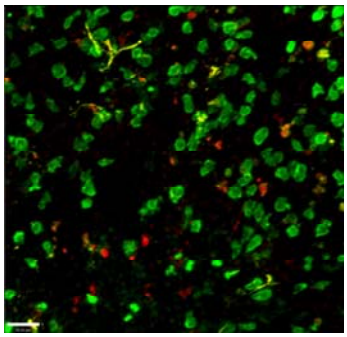
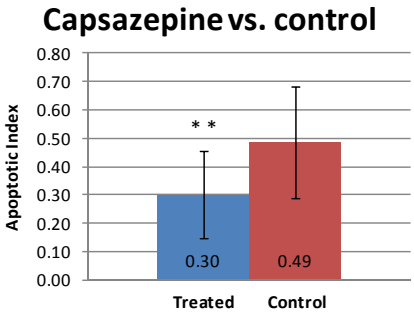
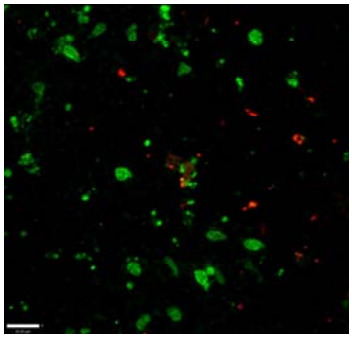


Figure 1 (upper left). Quantification of chick utriclar epithelium treated with $CuSO_4$ simultaneous with cisplatin administration (23 specimens, 41 images obtained) compared to control population (24 specimens, 46 images obtained) with same cisplatin dosage revealed statistically significant (<0.05) increase in apoptosis in treated cells. Image 1 (upper right). CuSO4 administration; hair cells labeled with Alexa 488 (green), apoptotic cells labeled with Cy3 (red). Image 2 (lower left). Control culture; hair cells labeled with Alexa 488 (green), apoptotic cells labeled with Cy3 (red).





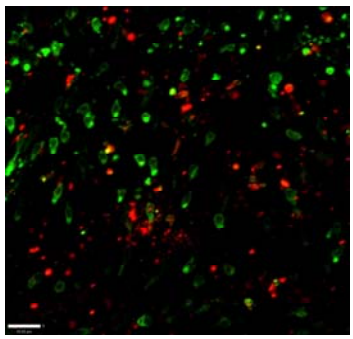


Figure 2 (upper left). Quantification of chick utriclar epithelium treated with capsazepine prior to cisplatin administration (11 specimens, 22 images obtained) compared to control (9 specimens, 17 images obtained) with same cisplatin dosage revealed statistically significant (<0.005) decrease in apoptosis in treated cells. Image 3 (upper right). Capsazepine administration; hair cells labeled with Alexa 488 (green), apoptotic cells labeled with Cy3 (red). Image 4 (lower left). Control culture; hair cells labeled with Alexa 488 (green), apoptotic cells labeled with Cy3 (red).

In contrast, application of capsazepine to post-hatch chick utricular epithelium prior to treatment with cisplatin caused a decrease in cell

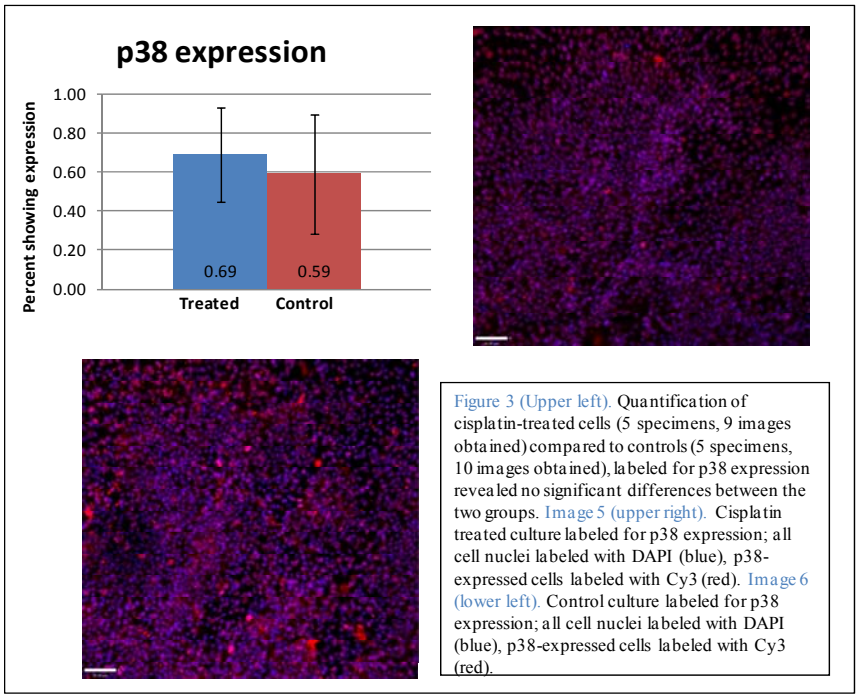


Figure 3 (Upper left). Quantification of cisplatin-treated cells (5 specimens, 9 images obtained) compared to controls (5 specimens, 10 images obtained), labeled for p38 expression revealed no significant differences between the two groups. Image 5 (upper right). Cisplatin treated culture labeled for p38 expression; all cell nuclei labeled with DAPI (blue), p38-expressed cells labeled with Cy3 (red). Image 6 (lower left). Control culture labeled for p38 expression; all cell nuclei labeled with DAPI (blue), p38-expressed cells labeled with Cy3 (red).

death compared to controls. While control cultures had a mean AI of 0.49 ±0.15, treated cultures had a significantly smaller mean AI of 0.30 ±0.20 (Figure 2, images 3 & 4). This was shown to be statistically significant to the <0.005 level with a Student's t-Test.

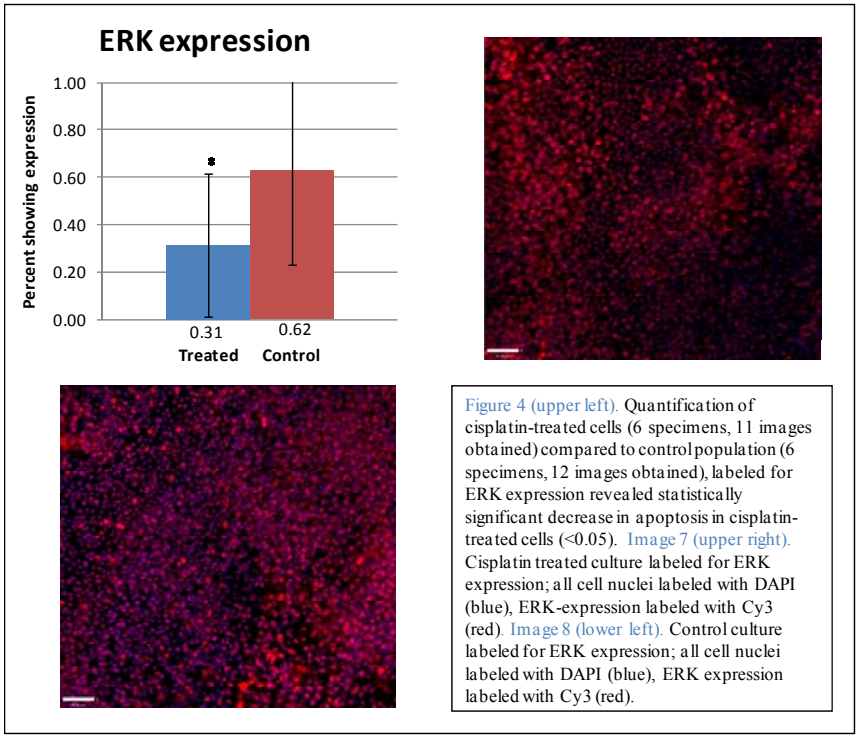
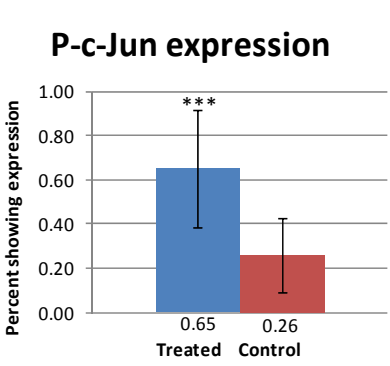
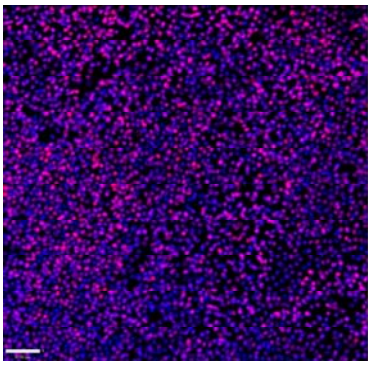


Figure 4 (upper left). Quantification of cisplatin-treated cells (6 specimens, 11 images obtained) compared to control population (6 specimens, 12 images obtained), labeled for ERK expression revealed statistically significant decrease in apoptosis in cisplatin-treated cells (<0.05). Image 7 (upper right). Cisplatin treated culture labeled for ERK expression; all cell nuclei labeled with DAPI (blue), ERK-expression labeled with Cy3 (red). Image 8 (lower left). Control culture labeled for ERK expression; all cell nuclei labeled with DAPI (blue), ERK expression labeled with Cy3 (red).

Following preliminary investigations of the effects of inhibiting c-Jun on apoptosis in the chick utricle, labeled cell counts were obtained for all cells with activated p38 and ERK1/2, as well as P-c-Jun. For P-c-Jun labeled cells, a labeled hair cell count was also obtained. Statistical analyses revealed a labeled cell count of 0.69 ±0.24 for cisplatin-treated cells labeled





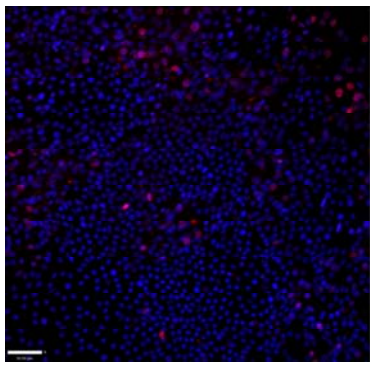
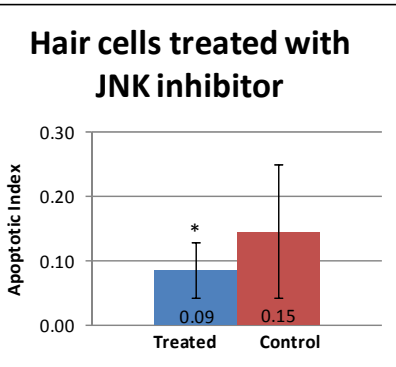
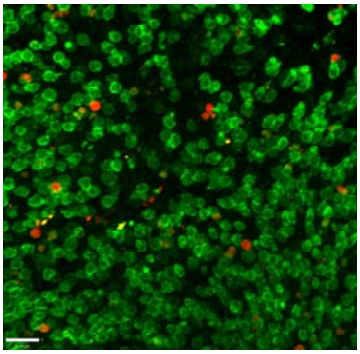


Figure 5 (upper left). Quantification of cisplatin-treated cells (7 specimens, 13 images obtained) compared to control cells (6 specimens, 12 images obtained) labeled for C-Jun expression revealed a statistically significant increase in cisplatin-treated cells (<0.0005). Image 9 (upper right). Cisplatin treated culture labeled for P-c-Jun expression; all cell nuclei labeled with DAPI (blue), P-c-Jun expressed cells labeled with Cy3 (red). Image 10 (lower right). Control culture labeled for P-c-Jun expression; all cell nuclei labeled with DAPI (blue), P-c-Jun expressed cells labeled with Cy3 (red).





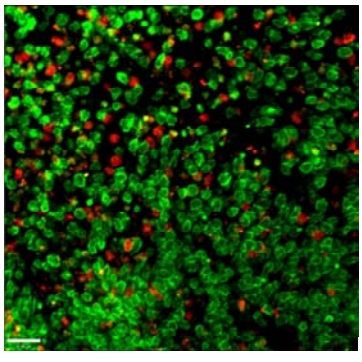
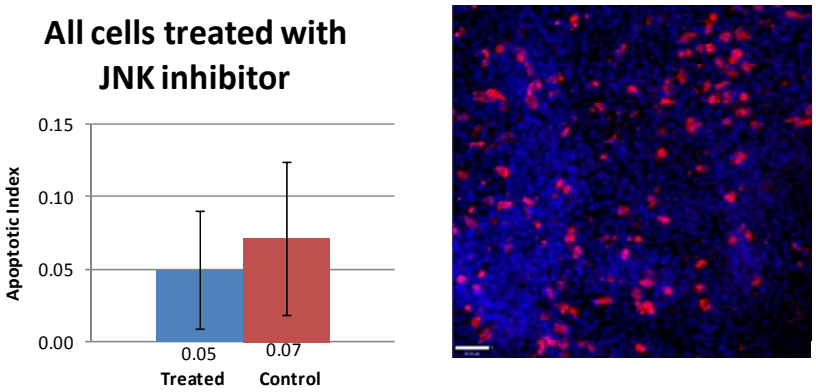


Figure 6 (Upper left). Quantification of chick utriclar cultures treated with JNK inhibitor and labeled for P-c-Jun expression (10 specimens, 19 images obtained) compared to control population (12 specimens, 22 images obtained) revealed statistically significant decrease in AI in treated population (<0.05). Image 11 (Upper right). Cisplatin treated culture with JNK inhibitor; hair cells labeled with Alexa 488 (green), apoptotic cells labeled with Cy3 (red). Image 12 (Lower left). Control culture treated with cisplatin; hair cells labeled with Alexa 488 (green), apoptotic cells labeled with Cy3 (red).

for p38 verses 0.59 ±0.30 for control cells. This was not statistically significant (Figure 3, images 5 & 6). For ERK-labeled cells treated with cisplatin, the calculated labeled cell count was 0.31 ±0.30 for treated cells, and 0.62 ±0.40 for control cells, which was statistically significant (<0.05) (Figure 4, images 7 & 8). c-Jun labeled cells treated with cisplatin revealed a labeled cell count of 0.65 ±0.27 versus 0.26 ±0.17 in control cells, this difference was significant (<0.0005) (Figure 5, images 9 & 10).

Based on these findings, we further investigated c-Jun's role in cisplatin toxicity. Inhibition of JNK resulted in a hair cell AI of 0.09

±0.04 for treated cultures, compared to a hair cell AI of 0.15 ±0.09 for control cultures, which was again statistically significant (<0.05) (Figure 6, images 11 & 12). Analysis of the non-hair cell specific AI yielded 0.05±0.04 for treated cells and 0.07±0.05 for control cells. This difference was not significant (Figure 7, images 13 & 14).



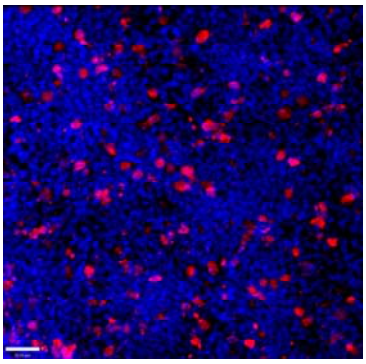


Figure 7 (Upper left). Quantification of cisplatin-treated chick utriclar cultures labeled for P-c-Jun expression and treated with JNK inhibitor (10 specimens, 19 images obtained) compared to control populations (12 specimens, 22 images obtained). Image 13 (upper right). Cisplatin treated cells with JNK inhibitor administration; all cell nuclei labeled with DAPI (blue), apoptotic cells labeled with Cy3 (red). Image 14 (lower left). Control cultures with cisplatin administration; all cell nuclei labeled with DAPI (blue), apoptotic cells labeled with Cy3 (red).

Discussion

Treatment with copper sulfate

Cisplatin-resistant cell lines have been noted to have cross-resistance to copper (Matsumoto et al., 2007). Interfering with copper transporters using $CuSO_4$ has been proposed to increase cisplatin intake into the cell. In the present study, $CuSO_4$ was administered simultaneously with cisplatin to cultures of chick utricular epithelium. Results agree with some previous studies, and showed increased apoptosis in $CuSO_4$-treated cells compared to controls. The present work extends previous findings in cell lines to avian utricles. Theories on the relation between increased $CuSO_4$ and apoptosis involve the transport of copper into and out of

the cell. Since increased levels of apoptosis likely indicate increased levels of cisplatin remain in the cell, it is possible that the ATP7A and ATP7B transporters are more efficient at removing cisplatin from the cell in control (i.e. non-$CuSO_4$-treated) cultures. It is likely that the influx of $CuSO_4$ may saturate the cell and subsequently reduce ATP7A and ATP7B-mediated efflux of cisplatin. Demonstration of a positive relationship between cell death and increased copper transport could potentially lead to the development of new therapeutic drugs to prevent cisplatin ototoxicity. Further research is required to confirm similar effects of $CuSO_4$ on avian utricle and mammalian utricle and cochlea.

TRPV1 inhibition

The second phase of the current study investigated the effects of inhibiting TRPV1 using the capsaicin antagonist capsazepine. Mukherjea et al. (2008) previously demonstrated that TRPV1 inhibition reduced apoptosis following cisplatin administration. Results from the current study agree with Mukherjea's data; avian utricular epithelia treated with capsazepine resulted in 30%±20% of hair cells undergoing apoptosis, compared to 49%±15% in control cultures. Because the TRPV1 gene is activated by multiple stimuli (both chemical and non-chemical), potential contraindications of inhibiting activation of the gene must be researched and considered. Further research applying *in-vitro* and *in-vivo* avian and mammalian models is needed to further explore the role of TRPV1 inhibition as a clinical strategy.

MAPK pathways

The third phase of the current study investigated the connection between MAPK pathways (p38, ERK1/2, JNK) and apoptosis following cisplatin administration. Identifying the level of MAPK activation in cisplatin-treated cells compared to controls revealed that 69% ±24%

of cisplatin-treated cells displayed activated p38, compared to 59% ±30% in control cells (not statistically significant); 31% ±30% of cisplatin-treated cells displayed activated ERK compared to 62% ±40% in control cells (statistically significant, <0.05); and 65% ±27% of cisplatin-treated cells displayed activated P-c-Jun compared to 26% ±17% in control cells (statistically significant, <0.0005). In addition, inhibiting JNK in cisplatin-treated cell cultures revealed 9% of hair cells undergoing apoptosis compared to 15% in control cultures (statistically significant, <0.05), and 5% of all cells (hair cells and supporting cells) were apoptotic in JNK-inhibited cultures, compared to 7% in control cell cultures (not statistically significant). The present results confirm previous results in other model systems demonstrating that inhibition of JNK decreases hair cell apoptosis (Eshraghi et al., 2007; Zine & Van De Water, 2004; Marderstein et al., 2003; Ylikoski et al., 2001; Pirvola, 2000; Tournier et al., 2000; Yang et al., 1997). Studies to date do not resolve whether the role of JNK is the same in hair cells and supporting cells. Further research should also address whether our observations in post-hatch chick utricle culture correspond to those in mammalian organ culture systems. Finally, given that we observed a significant decrease in apoptosis in cisplatin-treated ERK-labeled cells, future studies should investigate the relationship between inhibition of the ERK pathway and cisplatin treatment.

Conclusion

Multiple parallel cellular mechanisms mediate cisplatin ototoxicity. We show that $CuSO_4$, capsazepine, and SP600125 all significantly alter the amount of apoptosis in post-hatch chick utricle hair cells. While this is most certainly not an exhaustive list of potential pathways of cellular uptake, further investigation into these pathways will be essential to determinepossible clinical applications of the inhibitors tested above.

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